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(54) Title: PURIFICATION MEANS

(57) Abstract: Described is a novel purification tag, SNUT, based on the gene product of a sortase gene, in particular the *srtA* gene of *Staphylococcus aureus* and uses thereof. Also provided are expression constructs comprising DNA encoding the tag for the production of recombinant polypeptides. The tag can be used in methods of purifying soluble domains of a number of proteins previously not able to be isolated efficiently and methods of inducing and/or enhancing an immune response to an antigen of interest.

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1 Purification Means

2
3 The present invention relates to purification means,
4 in particular to means suitable for use in
5 purification of soluble proteins.

6

7 Introduction

8

9 The recombinant production of protein in bacteria,
10 yeast, insect and mammalian cell lines has become a
11 cornerstone of biological research and the
12 biotechnology industry. Classical biochemical and
13 chromatographical purification techniques usually
14 produce inadequate amounts of a target protein to
15 study its roles or actions. Even if enough of the
16 protein can be purified, it usually involves
17 cumbersome amounts of starting material or tissue
18 and many processing steps are taken before
19 reasonable purification can be achieved.

20

21 Recombinant expression of the target protein
22 bypasses a lot of these problems. By introducing

1 the target protein's gene template to a cell line or
2 bacterial culture, induced overexpression can result
3 in significant levels of that protein being
4 produced. Large amounts of protein make the
5 purification a lot simpler, but the addition or
6 fusion of purification domains or tags allows for a
7 relatively simple one-step purification using
8 affinity chromatography resins. However,
9 occasionally, due to the varying nature of proteins,
10 the production of soluble protein has remained
11 elusive with known tags unable to purify many
12 proteins. In some cases, production of protein can
13 be a problem due to differences in the machinery of
14 bacterial cells. There is therefore a need for a
15 more versatile tag than is available currently on
16 the market. The provision of such a versatile tag
17 enabling, for example, improved ability to quickly
18 produce and screen soluble protein in bacteria such
19 as *E.coli* would represent a major step forward in
20 protein biochemistry.

21

22 Summary of the Invention

23

24 The present inventors have developed a novel
25 purification tag based on the gene product of a
26 sortase gene, in particular the *srtA* gene of
27 *Staphylococcus aureus*. This tag, known as SNUT
28 [Solubility eNhancing Unique Tag] has been found to
29 have exceptional activity, enabling the efficient
30 purification of soluble domains of a number of
31 proteins hitherto not able to be isolated
32 efficiently using conventional purification tags.

1

2 Throughout this specification, reference to a SNUT
3 Tag should be understood to mean a tag derived from
4 a sortase gene product.

5

6 In a first aspect of the invention, there is
7 provided a purification tag comprising a sortase,
8 e.g srtA, gene product.

9

10 In preferred embodiments, the sortase gene product
11 is a gene product of the srtA gene of *Staphylococcus*
12 *aureus*.

13

14 Also provided is the use of a sortase, e.g srtA,
15 gene product as a purification tag.

16

17 Furthermore, according to a third aspect of the
18 invention, there is provided an expression construct
19 for the production of recombinant polypeptides,
20 which construct comprises an expression cassette
21 consisting of the following elements that are
22 operably linked: a) a promoter; b) the coding region
23 of a DNA encoding a sortase, eg srtA gene product as
24 a purification tag sequence; c) a cloning site for
25 receiving the coding region for the recombinant
26 polypeptide to be produced; and d) transcription
27 termination signals.

28

29 According to a fourth aspect of the invention, there
30 is provided a method for producing a polypeptide,
31 comprising: a) preparing an expression vector for
32 the polypeptide to be produced by cloning the coding

1 sequence for the polypeptide into the cloning site
2 of an expression construct according to the third
3 aspect of the invention; b) transforming a suitable
4 host cell with the expression construct thus
5 obtained; and c) culturing the host cell under
6 conditions allowing expression of a fusion
7 polypeptide consisting of the amino acid sequence of
8 the purification tag with the amino acid sequence of
9 the polypeptide to be expressed covalently linked
10 thereto; and, optionally, d) isolating the fusion
11 polypeptide from the host cell or the culture medium
12 by means of binding the fusion polypeptide present
13 therein through the amino acid sequence of the
14 purification tag.

15

16 The expression construct, herein referred to as
17 pSNUT, may be made by modification of any suitable
18 vector to include the coding region of a DNA
19 encoding a sortase. In preferred embodiments, the
20 expression construct is based on the pQE30 plasmid.

21

22 A sample of pSNUT was deposited with the National
23 Collections of Industrial and Marine Bacteria Ltd.
24 (NCIMB), 23 St Machar Drive, Aberdeen, Scotland AB24
25 3RY on 23 December 2002 under accession no NCIMB
26 41153.

27

28 In a fifth aspect, there is provided a fusion
29 polypeptide obtained by the method of the fourth
30 aspect of the invention.

31

1 The inventors have found that when a fusion
2 polypeptide comprising a polypeptide/protein of
3 interest and a SNUT tag is used as an antigen, the
4 immune response generated is significantly stronger
5 than that generated when the polypeptide/protein of
6 interest alone is used as the antigen.

7
8 Thus, in a sixth aspect of the present invention,
9 there is provided a method of inducing and/or
10 enhancing an immune response to an antigen of
11 interest, the method comprising administering the
12 antigen of interest with a sortase, e.g srtA, gene
13 product. The antigen of interest, which preferably
14 is a polypeptide/protein of interest, may be
15 administered simultaneously, separately or
16 sequentially with the sortase, e.g srtA, gene
17 product. In preferred embodiments, the antigen of
18 interest is linked to the sortase, e.g srtA, gene
19 product, preferably in the form of a fusion
20 polypeptide.

21
22 In a seventh aspect of the invention, there is
23 provided the use of a sortase, e.g srtA, gene
24 product as an immunogen. As with the sixth aspect,
25 the sortase, e.g srtA, gene product is preferably
26 administered as a fusion polypeptide comprising the
27 sortase, e.g srtA, gene product and an antigen of
28 interest.

29
30 In preferred embodiments, the sortase, e.g. srtA
31 gene product (SNUT) is encoded by the nucleotide
32 sequence shown in Figure 4 or a variant or fragment.

1 thereof. Preferably, the srtA gene product
2 comprises amino acids 26 to 171 of the SrtA sequence
3 shown in Figure 4 or a variant or fragment thereof.

4
5 Variants and fragments of and for use in the
6 invention preferably retain the functional
7 capability of the polypeptide i.e. ability to be
8 used as a purification tag. Such variants and
9 fragments which retain the function of the natural
10 polypeptides can be prepared according to methods
11 for altering polypeptide sequence known to one of
12 ordinary skill in the art such as are found in
13 references which compile such methods, e.g.
14 Molecular Cloning: A Laboratory Manual, J. Sambrook,
15 et al., eds., Second Edition, Cold Spring Harbor
16 Laboratory Press, Cold Spring Harbor, New York,
17 1989, or Current Protocols in Molecular Biology, F.
18 M. Ausubel, et al., eds., John Wiley & Sons, Inc.,
19 New York.

20
21 A variant nucleic acid molecule shares homology
22 with, or is identical to, all or part of the coding
23 sequence discussed above. Generally, variants may
24 encode, or be used to isolate or amplify nucleic
25 acids which encode, polypeptides which are capable
26 of ability to be used as a purification tag.

27
28 Variants of the present invention can be artificial
29 nucleic acids (i. e. containing sequences which have
30 not originated naturally) which can be prepared by
31 the skilled person in the light of the present
32 disclosure. Alternatively they may be novel,

1 naturally occurring, nucleic acids, which may be
2 isolatable using the sequences of the present
3 invention. Thus a variant may be a distinctive part
4 or fragment (however produced) corresponding to a
5 portion of the sequence provided in Figure 4. The
6 fragments may encode particular functional parts of
7 the polypeptide.

8
9 The fragments may have utility in probing for, or
10 amplifying, the sequence provided or closely related
11 ones.

12
13 Sequence variants which occur naturally may include
14 alleles or other homologues (which may include
15 polymorphisms or mutations at one or more bases).
16 Artificial variants (derivatives) may be prepared by
17 those skilled in the art, for instance by site
18 directed or random mutagenesis, or by direct
19 synthesis. Preferably the variant nucleic acid is
20 generated either directly or indirectly (e. g. via
21 one or amplification or replication steps) from an
22 original nucleic acid having all or part of the
23 sequences of Figure 4. Preferably it encodes a
24 polypeptide which can be used as a purification tag.

25
26 The term 'variant' nucleic acid as used herein
27 encompasses all of these possibilities. When used in
28 the context of polypeptides or proteins it indicates
29 the encoded expression product of the variant
30 nucleic acid.

31

1 Homology (i. e. similarity or identity) may be as
2 defined using sequence comparisons are made using
3 FASTA and FASTP (see Pearson & Lipman, 1988. Methods
4 in Enzymology 183 : 6398). Parameters are preferably
5 set, using the default matrix, as follows :
6 Gapopen (penalty for the first residue in a gap) :-
7 12 for proteins/-16 for DNA
8 Gapext (penalty for additional residues in a gap) :-
9 2 for proteins/-4 for DNA
10 KTUP word length : 2 for proteins/6 for DNA.
11 Homology may be at the nucleotide sequence and/or
12 encoded amino acid sequence level. Preferably, the
13 nucleic acid and/or amino acid sequence shares at
14 least about 60%, or 70%, or 80% homology, most
15 preferably at least about 90%, 95%, 96%, 97%, 98% or
16 99% homology with the sequence shown in Figure 4.
17
18 Thus a variant polypeptide in accordance with the
19 present invention may include within the sequence
20 shown in Figure 4, a single amino acid change or 2,
21 3, 4, 5, 6, 7, 8, or 9 changes, or about 10, 15, 20,
22 30, 40 or 50 changes. In addition to one or more
23 changes within the amino acid sequence shown, a
24 variant polypeptide may include additional amino
25 acids at the C terminus and/or N-terminus.
26
27 Naturally, regarding nucleic acid variants, changes
28 to the nucleic acid which make no difference to the
29 encoded polypeptide (i.e. 'degeneratively
30 equivalent') are included within the scope of the
31 present invention.
32

1 Preferred variants include one or more of the
2 following changes(using the annotation of AF162687):
3 nucleotide 604 AAG causing an amino acid mutation of
4 KAR; nucleotide 647 AAG, codon remains K, therefore
5 a silent mutation; nucleotide 982 GAA causing an
6 amino acid mutation of GAE.

7
8 Changes to a sequence, to produce a derivative, may
9 be by one or more of addition, insertion, deletion
10 or substitution of one or more nucleotides in the
11 nucleic acid, leading to the addition, insertion,
12 deletion or substitution of one or more amino acids
13 in the encoded polypeptide. Changes may be by way of
14 conservative variation, i. e. substitution of one
15 hydrophobic residue such as isoleucine, valine,
16 leucine or methionine for another, or the
17 substitution of one polar residue for another, such
18 as arginine for lysine, glutamic for aspartic acid,
19 or glutamine for asparagine. As is well known to
20 those skilled in the art, altering the primary
21 structure of a polypeptide by a conservative
22 substitution may not significantly alter the
23 activity of that peptide because the side-chain of
24 the amino acid which is inserted into the sequence
25 may be able to form similar bonds and contacts as
26 the side chain of the amino acid which has been
27 substituted out. This is so even when the
28 substitution is in a region which is critical in
29 determining the peptides conformation.

30
31 Also included are variants having non-conservative
32 substitutions. As is well known to those skilled in

1 the art, substitutions to regions of a peptide which
2 are not critical in determining its conformation may
3 not greatly affect its activity because they do not
4 greatly alter the peptide's three dimensional
5 structure.

6
7 In regions which are critical in determining the
8 peptides conformation or activity such changes may
9 confer advantageous properties on the polypeptide.
10 Indeed, changes such as those described above may
11 confer slightly advantageous properties on the
12 peptide e. g. altered stability or specificity.

13
14 SNUT tags and vectors may be used in methods of
15 purifying a soluble domain of a peptide.
16 Accordingly in a further aspect of the invention,
17 there is provided a method of producing a soluble
18 bioactive domain of a protein, the method
19 comprising the steps of cloning DNA encoding at
20 least one candidate soluble domain into at least one
21 expression vector, transfecting or transforming a
22 host cell with said vector, expressing said DNA in
23 said host cell, wherein said vector encodes a
24 sortase gene product.

25
26 The sortase gene product is preferably in the form
27 of a fusion protein.

28
29 The method may comprise the steps of analysis of DNA
30 coding for the protein of interest to identify
31 antigenic soluble domains, designing oligonucleotide
32 primers to amplify DNA encoding the domain,

1 amplifying DNA, cloning the DNA, optionally
2 screening clones for correct orientation of DNA,
3 expressing DNA in expression strains, analysing
4 expression products for solubility, analysing
5 products and production of soluble bioactive protein
6 domain.

7
8 The method optionally comprises the step of
9 producing a soluble bioactive protein domain of said
10 protein of interest.

11
12 The methods and tags of the invention may be used
13 with any suitable polypeptide/protein of interest,
14 for example for the purification of such
15 polypeptides/proteins of interest. As described
16 herein and exemplified in the following examples,
17 the inventors have demonstrated that the methods and
18 tags of the invention enable the efficient
19 purification of a large number of proteins, many
20 of which have not been amenable to efficient
21 isolation using conventional methods and tags.

22
23 In preferred embodiments of the invention, the
24 polypeptide/protein of interest is MAR1, Jak1 or
25 CD33, or a fragment thereof.

26
27 In particularly preferred embodiments, the
28 polypeptide/protein of interest is a variable domain
29 fragment e.g. a variable domain fragment of CD33.

30

1 Preferred features of each aspect of the invention
2 are as for each of the other aspects mutatis
3 mutandis.

4

5 The invention is exemplified with reference to the
6 following non limiting description and the
7 accompanying figures in which:

8

9 Figure 1 shows selected domains for amplification
10 from *in silico* analysis. Representation of a
11 candidate protein for the expression platform, in
12 this case Jak1 (human). Four fragments have been
13 chosen by analysis as depicted.

14

15 Figure 2 shows denaturing dot-blot analysis of
16 expression clones of fragments of MAR1 in pQE30.

17

18 Figure 3 shows a ribbon Diagram of *Staphylococcus*
19 *aureus* sortase. Ribbon diagram of the putative
20 structure of *S. aureus* SrtA protein (minus its N-
21 terminal membrane anchor). SNUT represents the
22 portion of this structure between the two yellow
23 arrows as shown. The yellow ball signifies a Ca^{2+}
24 ion, essential for the biological activity of this
25 protein. This diagram is taken from Hlangovan et
26 al., 2001, PNAS 98 (11) 6056
27 (doi:10.1073/pnas.101064198)

28

29 Figure 4 shows the Nucleotide Sequence and amino
30 acid sequence of SNUT fragment.

31

1 (a) This is the determined sequence of SNUT. The
2 fragment was cloned into pQE30 using the BamHI site
3 of this vector. When in the wanted orientation,
4 insertion results in the inactivation of the
5 upstream cloning site, therefore allowing any
6 subsequent cloning of target inserts with the
7 downstream BamHI site (see (b) for restriction map
8 of sequence).

9
10 Figure 5 illustrates qualitative purification
11 results using the SNUT fusion tag. (a) shows the
12 elution profile on SDS-PAGE of SNUT-Jak1 using AKTA
13 Prime native histag purification. Successful
14 elution of SNUT-Jak1 construct is signified by the
15 white arrow. (b) shows the elution profile on SDS-
16 PAGE of SNUT-MAR1 using AKTA Prime native histag
17 purification. Successful elution is shown by the
18 arrow. (c) shows the same gel stained in (b)
19 western blotted and detected using poly-histidine-
20 HRP antibody. This is confirmation that the eluted
21 species in (b) is actually SNUT-MAR1, of expected
22 molecular weight.

23
24 Figure 6 shows a Western blot of lysates using anti-
25 histag antibody.

26
27 Figure 7a illustrates the elution profile on SDS-
28 PAGE of SNUT-CD33.

29
30 Figure 7b illustrates a Western blot of the same gel
31 from Figure 7a using anti-histag antibody to detect
32 the proteins.

1 Figure 8a illustrates a Western blot using anti-
2 histag antibody to detect the proteins.

3

4 Figure 8b illustrates a Western blot of the same gel
5 as Figure 8b using anti-SrtA antibody to detect the
6 proteins.

7

8 Figure 8C shows a Western blot showing the detection
9 of the SNUT protein using an anti-SrtA monoclonal
10 antibody.

11

12 Template analysis and primer design

13

14 Analysis of the DNA coding for a protein of interest
15 may be performed using software packages such as
16 Vector NTI (Informax, USA) and
17 BLASTP (<http://www.ncbi.nlm.nih.gov/BLAST/>), p-fam (
18 www.sanger.ac.uk/pfam) and TM pred
19 (www.hgmp.mrc.ac.uk) which may be used to identify
20 complete domains within the protein that
21 significantly increase the likelihood of
22 antigenicity and/or solubility when expressed as a
23 subunit of the original protein coding sequence.

24

25 In order to increase the possibility of identifying
26 a soluble domain, preferably multiple sub-domains,
27 more preferably at least three sub-domains, for
28 example 3 to 9 sub-domains may be identified for
29 processing.

30

31 Oligonucleotide primers to amplify the selected sub-
32 domains may be designed with the help of

1 commercially available software packages such as the
2 internet software package Primer3 ([http://www-](http://www-genome.wi.mit.edu/genome_software/other/primer3.html)
3 [genome.wi.mit.edu/genome software/other/primer3.html](http://www-genome.wi.mit.edu/genome_software/other/primer3.html)
4 (Whitehead Institute for Biomedical Research),
5 Vector NTI (www.informaxinc.com) and DNASIS (Hitachi
6 Software Engineering Company (www.oligo.net)).

7
8 Typically primers for use in a method of the
9 invention are in the range 10-50 base pairs in
10 length, preferably 15 to 30, for example 20 base
11 pairs in length, with annealing temperatures in the
12 range 45-72°C, more conveniently 55-60°C. Primers
13 may be synthesised using standard techniques or may
14 be sourced from commercial suppliers such as
15 Invitrogen Life Technologies (Scotland) or MWG-
16 Biotech AG (Germany).

17

18 PCR of Insert

19

20 The desired inserts which encode the selected sub-
21 domains are amplified using the primers designed
22 specifically for that target gene using standard PCR
23 techniques. The template DNA for amplification can
24 be in the form of plasmid DNA, cDNA or genomic DNA,
25 depending on whatever is appropriate or indeed
26 available. Any suitable DNA polymerase may be used,
27 for example, Platinum Taq, Pfu (www.stratagene.com)
28 or Pfx (www.invitrogen.com). Any suitable PCR system
29 may be used, for example, the Expand High Fidelity
30 PCR system (Roche, Basel, Switzerland).

31

1 Several different thermocycler conditions may be
2 used with each set of primers. This increases the
3 chance of the PCR working without having to
4 individually optimise each new primer set. Typically
5 the following three programs may be used in the
6 method:

7

- 8 1. A standard PCR programme using the recommended
9 annealing temperature provided with the
10 primers.
- 11 2. A standard PCR programme using 50°C as the
12 temperature for annealing.
- 13 3. A touchdown PCR programme, where the annealing
14 temperature starts at a high temperature e.g
15 65°C for 10 cycles and then gradually decreases
16 the annealing temperature to 50°C over the
17 subsequent e.g 15 cycles.

18

19 Buffer conditions may be adjusted as required, for
20 example with respect to magnesium ion concentration
21 or addition of DMSO for the amplification of
22 difficult templates. Further details of a suitable
23 purification method which may be used with the
24 vector or tag of the invention can be found in our
25 co-pending PCT application PCT/GB02/05941, filed on
26 the same day as this application, published 24 July
27 2003, and claiming priority from GB 0131026.7.

28

29 The PCR products may be visualised using standard
30 techniques, for example on a 1.5% agarose gel
31 stained with Ethidium Bromide and the bands are cut

1 out of the gel and purified using Mini elute gel
2 extraction Kit (Qiagen, Crawley, England).

3

4 Expression Vectors

5

6 Amplified DNA inserts may be cloned into expression
7 vectors using techniques dictated by the multiple
8 cloning sites of the vector in question. Such
9 techniques are readily available to the skilled
10 person.

11

12 Any suitable expression system can be used in the
13 invention. Preferably, the expression system is
14 prokaryotic. Suitable vectors for use in the method
15 of the invention include any vector which can encode
16 SNUT [Solubility eNhancing Unique Tag], for example
17 pSNUT. This tag is based on the sequence of a trans-
18 peptidase found on the surface of gram-positive
19 bacteria. This protein is highly soluble, and
20 expressed as very high levels.

21

22 The inventors have found that SNUT is an ideal
23 fusion tag for conferring solubility and expression
24 levels to target protein fragments. SNUT may be
25 cloned into any suitable vector. For the purposes of
26 the examples shown in this application, the sequence
27 incorporating the SNUT fragment is cloned into pQE30
28 (Qiagen, Valencia, CA) in a manner allowing full use
29 of the multiple cloning site (MCS) of this vector
30 for downstream gene insertions.

31

1 Development of pSNUT

2

3 The inventors found that a tag based on the *srtA*
4 gene product from *Staphylococcus aureus* is highly
5 soluble, reacts well to purification schemes and
6 expresses particularly well. It was hypothesised
7 that the incorporation of a portion or domain of
8 this protein could represent a useful fusion tag in
9 the present method, and indeed the expression of any
10 poorly soluble protein in *E. coli*. Using NMR
11 studies, the 3D structure of this protein has been
12 predicted and is shown in Figure 3. We hypothesised
13 that by taking a portion of this structure, we could
14 make a manipulateable protein tag, but not disturb
15 its tertiary structure enough to reduce its highly
16 favourable characteristics listed above. The region
17 of this protein used as a solubility-enhancing tag
18 is depicted by two arrows.

19

20 The SNUT tag was cloned into pQE30. However, it may
21 be cloned into any suitable expression vector.
22 Positive clones may be identified by denaturing dot
23 blots, SDS-PAGE and Western blotting. Final
24 confirmation of these clones was provided by DNA
25 sequencing, and the sequence of the multiple cloning
26 region of the resultant vector is shown in Figure 4.

27

28 Variances in the sequence of the SNUT domain were
29 observed from the sequence for SrtA that has been
30 logged in Genbank (AF162687). The variances are
31 (using the annotation of AF162687) nucleotide 604
32 AAG causing an amino acid mutation of KAR;

1 nucleotide 647 AAG, codon remains K, therefore a
2 silent mutation; nucleotide 982 GAA causing an amino
3 acid mutation of GAE.

4
5 Preliminary trials and native purification showed
6 that the SNUT fragment was very soluble and its
7 characteristics were in no way diminished by
8 truncation, thus showing that SNUT could represent a
9 useful tag domain (data not shown). As described in
10 the Examples, to fully test the abilities of SNUT,
11 we then chose two proteins were soluble protein
12 production had proved impossible using conventional
13 methods and using the other expression systems of
14 the method of the present invention. Surprisingly,
15 we found that, using pSNUT in the method of the
16 invention, these proteins could be produced in
17 soluble form.

18

19 Clone Propagation

20

21 Target insert/expression vector ligations may be
22 propagated using standard transformation techniques
23 including the use of chemically competent cells or
24 electro-competent cells. The choice of the host
25 cell and strain for transformation is dependent on
26 the characteristics of the expression vectors being
27 utilised.

28

29 Bacterial cells, for example, *Escherichia coli*, are
30 the preferred host cells. However, any suitable
31 host cell may be used. In preferred embodiments, the
32 host cells are *Escherichia coli*.

1

2 The vectors may be used to each transfect or
3 transform a plurality of different host cell
4 strains. The set of host cell strains for
5 individual vector may be the same or different from
6 the set used with other vectors.

7

8 In a particularly preferred embodiment of the
9 invention, each vector may be transformed into three
10 *E. coli* strains (for example, selected from
11 Rosetta(DE3)pLacI, Tuner(DE3)pLacI, Origami BL21
12 (DE3)pLacI and TOP10F, Qiagen).

13

14 Where the vectors are pQE based vectors, TOP10F'
15 cells are preferred for the propagation and
16 expression trials of such vectors. The present
17 inventors have identified this strain as a more
18 superior strain for these vectors than either of the
19 recommended strains by the supplier (M15 and
20 SG13009), in terms of ease of use and culture
21 maintenance (only one antibiotic required as to two
22 with M15 or SG13009 (www.qiagen.com). Other F'
23 strains such as XL1 Blue can be used, but are
24 inferior to the TOP10F' strain, due to lack of
25 expression regulation (results not shown). The use
26 of TOP10F' (Invitrogen) for the propagation and/or
27 expression pQE based vectors forms an independent
28 aspect of the present invention. Other F' strains
29 such as XL1 Blue may also be used, but are inferior
30 to the TOP10F'.

31

1 After transformation, cells may be plated out onto
2 selection plates and propagated for the development
3 of single colonies using standard conditions.

4

5 Propagation of Cells

6

7 The colonies may be used to inoculate duplicate
8 wells in a 96 well plate.

9

10 Typically, each well may contain 200 µl of LB broth
11 with the appropriate antibiotics. Each plate may be
12 dedicated to one strain of *E. coli* or other host
13 cell which alleviates the problems of different
14 growth rates. The necessary controls are also
15 included on each plate. The plates are then grown
16 up, preferably at 37°C or any other temperature as
17 appropriate to the particular host cell and vector,
18 with shaking, until log phase is reached. This is
19 the primary plate.

20

21 From the primary plate a secondary plate is seeded
22 and then grown. Typically, the secondary plate is
23 be seeded using 'hedgehog' replicators and then
24 grown up to, for example, log phase, chilled to 16°C
25 for 1 hour. Determination of positive clones from
26 these plates may be undertaken using functional
27 studies. Routinely, 6-48 clones for each insert-
28 vector ligation are taken and propagated in culture
29 micro-titre plates containing up to 500 µl of media.
30 According to the conditions and reagents required,
31 protein production is then induced, and cultures
32 propagated further. Most vectors are under the

1 control of a promoter such as T7, T7lac or T5, and
2 can be easily induced with IPTG during log phase
3 growth. Typically, cultures are propagated in a
4 peptone-based media such as LB or 2YT supplemented
5 with the relevant antibiotic selection marker.
6 These cultures are grown at temperatures ranging
7 from 4-40 °C, but more frequently in the range of
8 20-37 °C depending on the nature of the expressed
9 protein, with or without shaking and induced when
10 appropriate with the inducing agent (usually log or
11 early stationary phase). After induction, growth
12 propagation can be continued for 1-16 hours for a
13 detectable amount of protein to be produced.
14
15 The primary plate is preferably stored at 4°C until
16 the process is complete.
17
18 **Colony Screening for Inserts in Correct Orientation**

1 The method of the invention may include the step of
2 testing transformants for correct orientation of the
3 inserts. Identification of positive clones can be
4 achieved through a variety of methods, including
5 standard techniques such as digestion analysis of
6 plasmid DNA; colony PCR and DNA sequencing.
7 Alternatively, dot-blotting may be used for the
8 identification of positive clones for example, using
9 a BioDot apparatus (BioRad) containing
10 nitrocellulose membrane (0.45µM pore size) in
11 accordance with the manufacturers' instructions,
12 prior to final confirmation by DNA sequencing.

13
14 The use of this dot blotting method in the platform
15 represents a rapid, reproducible and robust
16 detection method. This particular method is useful
17 for the rapid detection or presence of recombinant
18 protein and allows for a determination of all clones
19 irrespective of solubility and conformation. This
20 may be important at this stage, because
21 conformational structures can inhibit the detection
22 of tag domains if they are not presented properly on
23 the surface of the protein. This can occur as
24 easily with both soluble and insoluble protein.

25
26 As described above, standard colony PCR techniques
27 may be used. For example, transformants may be
28 selected, either manually or using automation such
29 as the Cambridge BioRobotics BioPick instrument, and
30 screened using directional PCR using a primer that
31 encodes for a sequence on the vector such as S Tag
32 or GATA sequence, and then the complementary primer

1 from the insert. A PCR mix may be used such as the
2 RedTaq DNA Polymerase (Sigma Aldrich, Dorset,
3 England) and the thermocycler conditions used may be
4 the standard PCR programme using 50°C as the
5 annealing temperature or adjusted as required.

6

7 Although all colony selecting and picking can be
8 done manually, automated colony pickers are
9 preferred. Automated colony pickers such as the
10 BioRobotics BioPick allow for the uniform and
11 reproducible selection of clones from transformation
12 plates. Clone selection determinants can be set to
13 ensure picking colonies of a standardised size and
14 shape. After picking and plate inoculation,
15 propagation of clones can be carried out as
16 described above.

17

18 Identification of positive clones can be achieved
19 through a variety of methods, including standard
20 techniques such as digestion analysis of plasmid
21 DNA; colony PCR and DNA sequencing. Alternatively, in
22 a preferred embodiment, the novel method of dot-
23 blotting described herein for the identification of
24 positive clones may be used in place of such
25 traditional techniques, prior to final confirmation
26 by DNA sequencing. The use of this method in the
27 platform presented here is not essential in the use
28 of this platform over existing screening
29 methodologies, but represents a rapid, reproducible
30 and robust detection method. The protocol described
31 here is a new protocol for an existing method for

1 which commercially available equipment (Bio-Rad
2 DotBlot) can be purchased.
3
4 This particular method is useful for the rapid
5 detection or presence of recombinant protein and
6 allows for a determination of all clones
7 irrespective of solubility and conformation. This
8 is useful at this stage, because conformational
9 structures can inhibit the detection of tag domains
10 if they are not presented properly on the surface of
11 the protein. This can occur as easily with both
12 soluble and insoluble protein.
13
14 For example, after growth on the micro-titre plates
15 is complete, the plate is centrifuged at 4000 rpm
16 for 10 minutes at 4°C to harvest the bacterial
17 cells. The supernatant is removed and the cell
18 pellets are re-suspended in 50 µl lysis buffer (10
19 mM Tris.HCl, pH 9.0, 1mM EDTA, 6 mM MgCl₂)
20 containing benzonase (1 µl/ml). The plate is
21 subsequently incubated at 4°C with shaking for 30
22 minutes. A sample (10 µl) of the cell lysate is
23 added to 100 µl buffer (8 M urea, 500 mM NaCl, 20 mM
24 sodium phosphate, pH 8.0) and incubated at room
25 temperature for 20 minutes. Samples are then
26 applied to a BioDot apparatus (BioRad) containing
27 nitrocellulose membrane (0.45µm pore size) in
28 accordance with the manufacturers' instructions.
29 The membrane is removed and transferred into
30 blocking reagent (3% w/v; Bovine serum albumin in
31 TBS) for 30 minutes at room temperature. The blot
32 is washed briefly with TBS then incubated in a

1 primary antibody, specific to the tag being used for
2 the subset of expression clones. Depending on the
3 nature of the primary i.e., whether or not it has a
4 horse radish peroxidase (HRP) reporter function,
5 will depend on whether the use of a secondary is
6 required. For detection of specific binding the
7 membrane is then washed 2x 5 minutes in TBS followed
8 by 1x 5 minute wash in 10 mM Tris.HCl pH7.6.
9 Detection of specifically bound antibody is
10 disclosed by the addition of chromogenic substrate
11 (6 mg diaminobenzidine in 10 ml 10 mM Tris.HCl pH
12 7.6 containing 50 μ l 6% H₂O₂) . The reaction is
13 stopped by thorough rinsing in water. Positive
14 clones identified by this procedure can then be
15 confirmed by DNA sequencing of the expression
16 construct using now industry-standard techniques and
17 equipment such as ABI and Amersham Biosciences.

18

19 Sequencing

20

21 The sequencing reactions may be performed using
22 techniques common in the art using any suitable
23 apparatus. For example, sequencing may be performed
24 on the cloned inserts, using the Big Dye Terminator
25 cycle sequencing kits (Applied Biosystems,
26 Warrington, UK) and the specific sequencing primer
27 run on a Peltier Thermal cycler model PTC225 (MJ
28 Research Cambridge, Mass). The reactions may be run
29 on Applied Biosystems - Hitachi 3310 Sequencer
30 according to the manufacturer's instructions. These
31 sequences are checked to ensure that no PCR
32 generated errors have occurred.

1

2 **Assessment of Solubility of Positive Clones**

3

4 The cells of positive clones may be harvested and
5 soluble and insoluble protein detected.

6

7 Any suitable techniques known in the art can be used
8 to separate soluble and insoluble protein, such as
9 the use of centrifugation, magnetic bead
10 technologies and vacuum manifold filtrations.
11 Typically, however, the separated proteins are
12 ultimately analysed by acrylamide gel and western
13 blotting. This confirms the presence of recombinant
14 protein at the correct size.

15

16 In one embodiment, contents of each well in the 96
17 well plate are transferred into a Millipore 0.65 μ m
18 multi-screen plate. The plate is placed on a vacuum
19 manifold and a vacuum is applied. This draws off
20 the culture medium to waste. The cells are then
21 washed with PBS (optional), again the vacuum is
22 applied to remove the PBS. The multi-screen plate is
23 removed from the manifold and bacterial cell lysis
24 buffer (containing DNase) (50 μ l) is added to each
25 well. The plate is incubated at room temperature
26 for 30 minutes with shaking to facilitate lysis of
27 the cells. A fresh 96 well microtitre plate (ELISA
28 grade) is placed inside the vacuum manifold and the
29 multi-screen plate is placed above it. When a
30 vacuum is applied the contents of each well are
31 drawn into the micro-titre plate below. The vacuum
32 only needs to be applied for 20 seconds. The

1 collected lysate contains the soluble fraction of
2 expressed protein. A sample of the collected lysate
3 may subsequently analysed by SDS-PAGE and Western
4 blotting to confirm both the presence and correct
5 molecular weight of the target protein.

6
7 The use of SDS-PAGE and Western blotting can be
8 expensive and time consuming, especially when
9 numerous samples must be analysed for each
10 construct. In light of this we have developed a
11 protocol whereby one gel can be used for both total
12 protein staining and western blotting. This
13 represents a significant improvement in this
14 methodology and obviously allows cost saving, and
15 precise comparisons can be made with regard to total
16 protein and western blotting as both sets of results
17 come from the one gel.

18
19 The basis of this protocol is in the ability to use
20 chloroform and UV light to stain protein on an SDS-
21 PAGE gel (Kazmin et al., Anal Biochem, 2001, 301(1)
22 91-6; doi:10.1006/abio.2001.5488). We have used
23 this technique to great effect as it allows for the
24 extremely rapid staining of a SDS-PAGE gel in less
25 than a tenth of the time taken using other more
26 traditional staining methods such as Commassie
27 Brilliant Blue and Collodial Blue stains. We then
28 decided to take this observation a step further and
29 analyse the ability of a chloroform-stained gel to
30 be used in Western blotting. This would not be
31 expected to work as other stained gels result in the
32 fixing of the protein to the gel and subsequent

1 inability to transfer the protein during blotting.
2 This expectation is coupled to the fact that
3 chloroform is not compatible with western blotting
4 equipment (Bio-Rad SD blotter user's manual).
5 However, fortuitously, we have discovered that with
6 a wash of the chloroform-stained gel in double-
7 distilled water, to remove excess chloroform, and
8 after subsequent soaking in transfer buffer,
9 proteins were effectively transferred during western
10 blotting in contrast to expectations. This transfer
11 was no-less effective than from a gel that has not
12 been pre-stained with chloroform and UV light.
13 Figure 6 primarily shows results relating to the
14 production of soluble protein by the platform, but
15 also shows the ability to use the chloroform-stained
16 SDS-PAGE derived western blot for the identification
17 of proteins, without any apparent damage caused to
18 the proteins.

19

20 The use of a chloroform-stained SDS-PAGE derived
21 western blot for the identification of proteins
22 forms another aspect of the present invention.

23

24 Scale-Up and Purification

25

26 This analysis provides a picture of the expression
27 status of the clones on each plate. Using this
28 analysis, positive soluble protein expressing clones
29 can be identified for the production of soluble
30 recombinant protein for a given target protein. The
31 clones may be selected and their growth scaled up
32 e.g. to 5 ml scale, using the saved primary plate as

1 an inoculum. Parameters that may be taken into
2 consideration in deciding on the appropriate culture
3 to select for scale-up include the desirability of
4 specific regions for the production of an antigen,
5 the overall expression levels of the clone and
6 factors that may affect affinity purification such
7 as amino acid composition.

8

9

10 Examples

11

12 Example 1. Expression construct design

13

14 Figure 1 is a diagrammatic representation of the
15 protein Jak1. Using pfam, the position of distinct
16 domains was established. Further analysis of these
17 domains was then carried out using Tmpred and the
18 Kyle and Dolittle hydrophobicity algorithm to
19 determine the usefulness of these domains as soluble
20 antigens. From this tentative analysis, four
21 domains were selected for amplification and
22 expression analysis. Based on this preliminary in
23 silico analysis, primers specific for a target
24 protein were designed and used to amplify domains
25 selected for analysis.

26

27 Vectors (500 ng) were restricted with *Bam*HI (20
28 units) and *Sal*I (20 units) in the presence of calf
29 intestinal alkaline phosphatase (CIP) (2 units), gel
30 purified and quantified using standard methods.
31 Purified PCR fragments (100 ng) were restricted with
32 *Bam*HI (5 units) and *Sal*I 5 units), gel purified,

1 quantified, and then used in a ligation reaction
2 with the restricted vector again using standard T4
3 DNA ligase methods (Ready-to-Go T4 DNA ligase,
4 Amersham Biosciences). A sample of the ligation
5 reaction (1 µl) was then used to transform the
6 appropriate competent bacterial cells (TOP10F' were
7 used here for the pQE based vectors, a modification
8 of the manufacturers recommendations; BL21(DE3)pLysE
9 for pET43.1a and TOP10F' for pGEX-Fus).
10 Transformants were selected on LB/ampicillin (100
11 µg/ml) overnight at 28°C.

12
13 A Cambridge BioRobotics BioPick instrument was used
14 for the picking of 24 colonies from each of the
15 transformant plates into flat-bottomed and lidded
16 micro-titre plates. The clones were used to
17 inoculate 150 µl of LB (containing 100µg/ml
18 ampicillin), and these were allowed to grow
19 overnight at 37 °C.

20
21 A secondary plate was prepared by the inoculation of
22 200 µl of LB containing the required supplements
23 with 10 µl of the overnight primary culture. These
24 were then grown at 37 °C Once an optical density
25 (OD) of 0.25 at A550 was reached, IPTG (final
26 concentration, 1 mM) was added to induce expression
27 of the recombinant protein. Culture propagation was
28 continued for another 4 hours prior to harvesting of
29 bacterial cells.

30
31 After clones expressing specific recombinant protein
32 have been identified, the solubility of these

1 proteins has to be established prior to clone
2 selection for purification. This can be performed a
3 number of ways including the use of centrifugation
4 and automation-friendly vacuum manifold separations.
5 The results here were obtained using methodologies
6 based around the use of vacuum-assisted filtration
7 to separate soluble and insoluble protein. The
8 filtrates that were produced from the method
9 described were then analysed by SDS-PAGE and Western
10 blotting to confirm the production of a recombinant
11 protein of the correct anticipated molecular weight.

12

13 Example 2 Design and Construction of SNUT Expression 14 Tag

15

16 Based on analysis of the amino acid sequence and
17 predicted structure of SrtA_{AN}, it was decided to
18 amplify the region of amino acids 26 to 171 of the
19 SrtA sequence. Amplification was conducted using
20 the forward primer 5' TTTTITAGATCTAAACCACATATCGAT
21 and the reverse primer 5'
22 TTTTITGGATCCATCTAGAACTTCTAC. This product was then
23 digested with *Bgl*I and *Bam*HI and ligated into pQE30
24 vector which had also been digested with *Bam*HI to
25 form the pSNUT vector. The ligation mix was
26 transformed into TOP10F' cells and single colonies
27 propagated on LB agar containing 100 µg/ml
28 ampicillin. Clones with the *srtA* fragment in the
29 correct orientation were screened by expression
30 analysis and positive clones identified using the
31 denaturing dot-blot assay described earlier.

32

1 The sequence encoding the SNUT tag was cloned into
2 pQE30 as described earlier and positive clones
3 identified by denaturing dot blots, SDS-PAGE and
4 Western blotting. Final confirmation of these
5 clones was provided by DNA sequencing, and the
6 sequence of the multiple cloning region of the
7 resultant vector is shown in Figure 4. Variances in
8 the sequence of the SNUT domain were observed from
9 the sequence for SrtA that has been logged in
10 Genbank (AF162687). The variances are (using the
11 annotation of AF162687) nucleotide 604 AAG causing
12 an amino acid mutation of KAR; nucleotide 647 AAG,
13 codon remains K, therefore a silent mutation;
14 nucleotide 982 GAA causing an amino acid mutation of
15 GAE.

17 Example 3 Trials of SNUT Expression Constructs

19 Target inserts were cloned into the pSNUT vector
20 using primer construction and digestion of resulting
21 PCR amplifications with *Bam*HI and *Sal*I as described
22 earlier. pSNUT was digested with *Bam*HI in a similar
23 manner and the target inserts cloned as described.
24 Clones were screened using the denaturing dot-blot
25 system and then analysed with SDS-PAGE and western
26 blotting. Positive clones were used for preparative
27 200 ml LB cultures containing 100 µg/ml ampicillin
28 and induced as described earlier. This was grown to
29 an optical density of 0.5 at A₅₅₀ at 37 °C.
30 Expression of SNUT was then induced with the
31 addition of IPTG (final concentration, 1 mM) and

1 left to grow for another 4 hours. Cells were then
2 harvested by centrifugation at 5K rpm for 15
3 minutes. Cells were re-suspended in 30 ml PBS
4 containing 0.1% Igepal and lysis induced by two
5 freeze-thaw cycles. The suspension was then
6 sonicated and centrifuged at 5K rpm for 15 minutes.
7 The soluble supernatant was transferred to a fresh
8 container and filtered through a 0.8 μ m disc filter
9 to remove final cell debris. This solution was then
10 applied to a Ni²⁺ charged IMAC column (Amersham
11 Biosciences HiTrap Chelating column, 1 ml) using an
12 AKTA Prime low pressure chromatography system and
13 column was then treated using a standard native his-
14 tag purification protocol involving washing of
15 column with 20 mM sodium dihydrogen phosphate pH 8.0
16 containing 10 mM imidazole, 500 mM NaCl, and elution
17 of soluble his-tagged proteins using 20 mM sodium
18 dihydrogen phosphate pH 8.0 containing 500 mM
19 imidazole, 500 mM NaCl. Elution fractions were then
20 analysed on an SDS-PAGE gel (4-20% SDS-PAGE Bio-Rad
21 Criterion gel), which was stained with chloroform as
22 described earlier. This gel was then subsequently
23 western blotted and the his-tagged protein detected
24 with anti-poly-histidine monoclonal antibody using
25 the techniques described herein.

26

27 Preliminary trials and native purification showed
28 that the SNUT fragment was very soluble and its
29 characteristics were in no way diminished by
30 truncation, thus showing that SNUT could represent a
31 useful tag domain (data not shown). To fully test
32 the abilities of SNUT, we then chose two proteins

1 for which soluble protein production had proved
2 impossible using the other expression systems in
3 which SNUT was not used as a tag. These were murine
4 MAR1 and human Jak1. Clones were prepared and
5 selected using the method as described in the
6 Examples above and positive clones were subsequently
7 grown and induced at 37 °C. These were then treated
8 to identical native histag purifications. Both
9 proteins behaved very favourably under standard
10 purification conditions as can be seen from the
11 purification profiles in Figure 5. For both these
12 trial proteins, this was the first example of such
13 purification under soluble conditions. The
14 production of these proteins using conventional
15 techniques has failed to produce any soluble
16 protein, irrespective of expression system or growth
17 conditions used (data not shown). However, as
18 described in this example, when the protein
19 fragments were expressed in pSNUT, soluble proteins
20 can be surprisingly obtained.

21
22 The effectiveness of SNUT as a fusion protein is
23 even more significant when it is considered that no
24 special growth conditions were required for the
25 generation of soluble protein. This is remarkable
26 when one considers the protein expressionist's
27 standard GST tag which is not even soluble itself
28 when expressed at 37 °C; 28 °C is required before
29 even the generation of GST on its own without any
30 target protein is observed.

31

1 Example 4 Purification of CD33 fragments using SNUT

2 Expression Constructs

3

4 Cloning Results

5

6 CD33 contains two extracellular immunoglobulin
7 domains. The extracellular region of the CD33 DNA
8 sequence had been cloned into several vectors for
9 expression, including expression as a fusion tag to
10 DHFR and NusA. None of these vectors produced
11 recombinant CD33 protein. The CD33 extracellular
12 region was also cloned into pSNUT. Both pSNUT and
13 CD33 were restricted with BamHI and HindIII under
14 standard conditions and ligated together using T4
15 DNA ligase, again under standard manufacturer's
16 protocols. TOP10F' cells were transformed with the
17 ligation product.

18

19 6 colonies were picked from the transformation plate
20 and grown in 150µl LB in a 96-well plate at 37°C
21 overnight

22

23 Expression analysis:

24

25 The overnight cultures were used to inoculate fresh
26 LB cultures (10µl into 190µl LB + 50µg/ml
27 ampicillin) and grown at 37°C for 2 hours.
28 Expression of the SNUT-CD33 construct was induced
29 with 1mM IPTG.

30

31 Cells were pelleted after 4 hours and lysed in PBS +
32 0.1% Igepal. Lysates were analysed by western blot

1 using anti-histag antibody. As shown in Figure 6,
2 it was clear that colonies 1, 3 and 4 were positive
3 and 2 was not (SNUT only).

4

5 Large Scale Expression:

6

7 The clone pertaining to lane 1 of Figure 6 was
8 chosen for sequencing analysis, which proved
9 successful insertion into the pSNUT vector. This
10 clone was grown in large scale (200ml) for
11 expression of the SNUT-CD33 construct at 37°C.
12 Expression was induced whenever the OD₆₀₀=0.4-0.6.
13 After 4-6 hours expression, the cells were pelleted
14 and lysed in 8M urea buffer. Lysates were clarified
15 and purified by immobilised metal affinity
16 chromatography (IMAC) using a re-folding technique
17 of decreasing urea concentration. At 0M urea, the
18 SNUT-CD33 was eluted from the IMAC column and
19 analysed by SDS PAGE using Coomassie blue stain
20 (Figure 7A) and Western Blotting (Figure 7B) using
21 anti-histag antibody.

22

23 Antibody Detection of expressed protein:

24

25 The SNUT fusion protein contains an N-terminal His-
26 tag. This facilitates detection using commercially
27 available anti-His antibodies, and can be used as a
28 means for purification of the recombinant protein
29 via IMAC as described (see Figure 8a).

30

31 In addition, we have developed in-house a polyclonal
32 antibody against SNUT and it also provides a

1 detection and purification means, as demonstrated in
2 Figure 8b.

3

4 Furthermore, the inventor has developed monoclonal
5 antibodies against SNUT which may also be used in
6 detection and purification methods of the invention.
7 A hybridoma producing monoclonal antibodies against
8 SNUT was developed as follows:

9

10 4 BALB/c mice were immunised intraperitoneally with
11 a purified SNUT recombinant protein. Seven
12 inoculations of 50µl of the antigen mixed with 50µl
13 of adjuvant were given over a ten-week time course.
14 Test bleeds were taken at intervals and positive
15 immunisation was confirmed by Western blot. Two days
16 after final inoculation, the mouse spleen cells were
17 fused with SP2 myeloma cells. The resulting
18 hybridoma cells were maintained in HAT media.
19 Microtitre plates were coated with the immunising
20 antigen (50ng/well) together with a control. Eleven
21 days post fusion actively growing Hybridoma cells
22 were ELISA screened for specificity to SNUT. Those
23 giving high readings were cloned twice by limiting
24 dilutions. An ECL of supernatant was performed as a
25 final control of their specificity.

26

27 Figure 8C shows a Western blot showing the detection
28 of the SNUT protein using one of the monoclonal
29 antibodies developed.

30

31 Results:

32

1 CD33 has been a very difficult protein to express.
2 The most desirable part of the protein for antigen
3 production is the extracellular variable domain.
4 There are two immunoglobulin domains in the
5 extracellular region of CD33, a membrane distal
6 variable (IgV) domain and a membrane proximal
7 constant (C2) domain. Expression analysis had been
8 performed for three fragments of the extracellular
9 region: the variable domain, the constant domain and
10 the full extracellular region in a number of
11 commercially available expression vectors. Only the
12 constant domain fragment would express in any of the
13 vectors. In order to express the desired variable
14 domain, the full length extracellular fragment and
15 the IgV domain fragment were cloned into our pSNUT
16 vector. Expression was successful for the full
17 length fragment.

18

19 The full length fragment was also purified
20 successfully by re-folding on an IMAC column. Not
21 only has the pSNUT vector allowed us to express a
22 protein fragment that has been unable to be
23 expressed in any tried commercially available
24 vector, including vectors with fusion tags designed
25 to increase expression such as NusA and DHFR, but
26 has allowed us to purify the expressed protein using
27 immobilised metal affinity chromatography by
28 standard techniques, and can be used for detection
29 of any protein expressed in the vector using either
30 anti-His or anti-SrtA antibodies.

31

1 All documents referred to in this specification are
2 herein incorporated by reference. Various
3 modifications and variations to the described
4 embodiments of the inventions will be apparent to
5 those skilled in the art without departing from the
6 scope and spirit of the invention. Although the
7 invention has been described in connection with
8 specific preferred embodiments, it should be
9 understood that the invention as claimed should not
10 be unduly limited to such specific embodiments.
11 Indeed, various modifications of the described modes
12 of carrying out the invention which are obvious to
13 those skilled in the art are intended to be covered
14 by the present invention.
15

1 Claims

2

3 1. Use of a sortase gene product as a purification
4 tag.

5

6 2. Use of a sortase, e.g srtA, gene product as an
7 immunogen.

8

9 3. The use according to claim 1 or claim 2 wherein
10 the sortase gene product is a Staphylococcus
11 aureus srtA gene product.

12

13 4. The use according to any one of claims 1 to 3
14 wherein the sortase gene product is encoded by
15 the nucleotide sequence shown in Figure 4 or a
16 variant or fragment thereof.

17

18 5. The use according to any one of claims 1 to 4
19 wherein the sortase gene product comprises
20 amino acids 26 to 171 of the SrtA sequence
21 shown in Figure 4 or a variant or fragment
22 thereof.

23

24 6. An expression construct for the production of
25 recombinant polypeptides, which construct
26 comprises an expression cassette consisting of
27 the following elements that are operably
28 linked: a) a promoter; b) the coding region of
29 a DNA encoding a sortase gene product as a
30 purification tag sequence; and c) a cloning
31 site for receiving the coding region for the

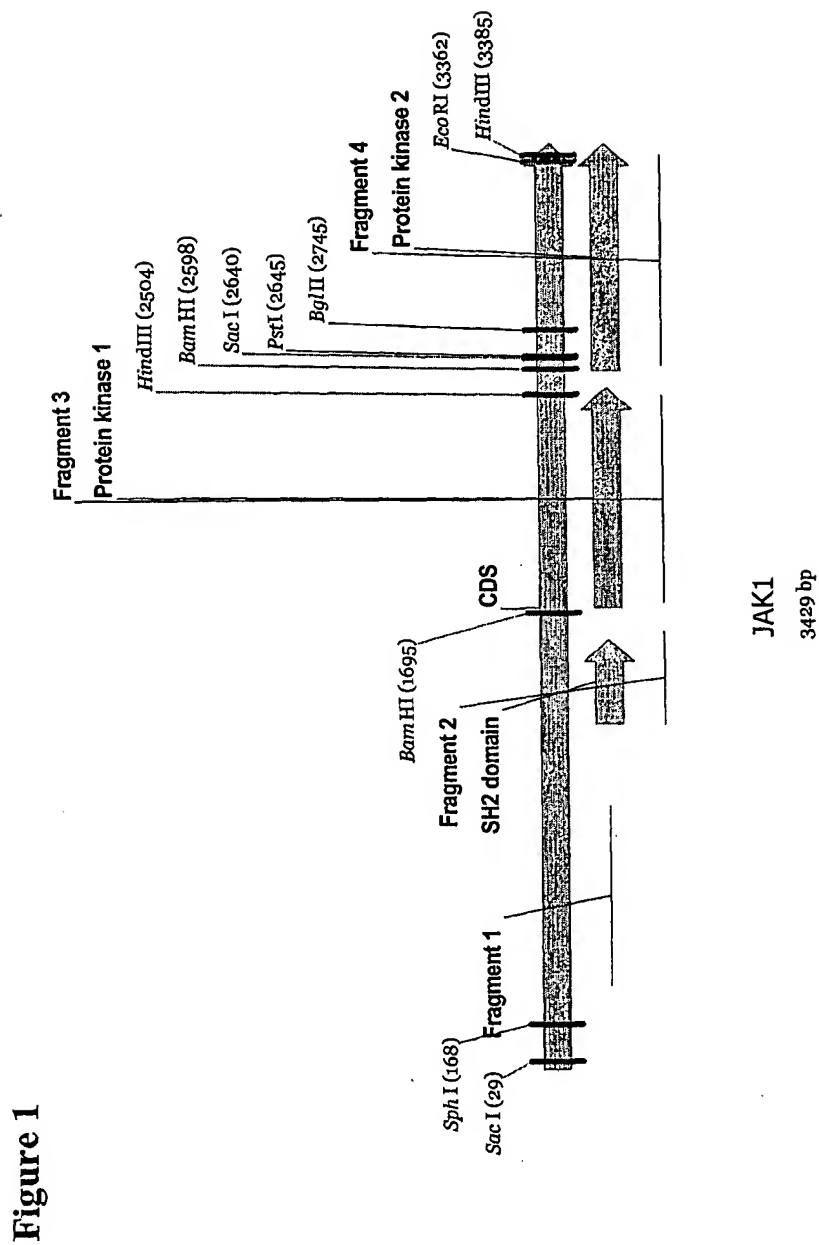
- 1 recombinant polypeptide to be produced; and d)
2 transcription termination signals.
3
- 4 7. The expression construct according to claim 6
5 wherein the sortase gene product is a
6 *Staphylococcus aureus* srtA gene product.
7
- 8 8. The expression construct according to claim 6
9 or claim 7 wherein the sortase gene product is
10 encoded by the nucleotide sequence shown in
11 Figure 4 or a variant or fragment thereof.
12
- 13 9. The expression construct according to any one
14 of claims 6 to 8 wherein the sortase gene
15 product comprises amino acids 26 to 171 of the
16 SrtA sequence shown in Figure 4 or a variant or
17 fragment thereof.
18
- 19 10. A method for producing a polypeptide,
20 comprising:
21 a) preparing an expression vector for the
22 polypeptide to be produced by cloning the
23 coding sequence for the polypeptide into the
24 cloning site of an expression construct as
25 claimed in any one of claims 6 to 9;
26 b) transforming a suitable host cell with the
27 expression construct thus obtained; and
28 c) culturing the host cell under conditions
29 allowing expression of a fusion polypeptide
30 consisting of the amino acid sequence of the
31 purification tag with the amino acid sequence

- 1 of the polypeptide to be expressed covalently
2 linked thereto; and d) isolating the fusion
3 polypeptide from the host cell or the culture
4 medium by means of binding the fusion
5 polypeptide present therein through the amino
6 acid sequence of the purification tag.
7
- 8 11. The method according to claim 10, wherein the
9 sortase gene product is a *Staphylococcus aureus*
10 *srtA* gene product.
11
- 12 12. The method according to claim 10 or claim 11
13 wherein the sortase gene product is encoded by
14 the nucleotide sequence shown in Figure 4 or a
15 variant or fragment thereof.
16
- 17 13. The method according to any one of claims 10 to
18 12 wherein the sortase gene product comprises
19 amino acids 26 to 171 of the *SrtA* sequence
20 shown in Figure 4 or a variant or fragment
21 thereof.
22
- 23 14. A fusion polypeptide obtained by the method of
24 any one of claims 10 to 13.
25
- 26 15. A purification tag comprising a sortase gene
27 product.
28
- 29
- 30 16. The purification tag according to claim 15
31 wherein the gene product is a *Staphylococcus*

- 1 aureus srtA gene product.
2
3
- 4 17. The purification tag according to claim 15 or
5 claim 16 wherein the sortase gene product is
6 encoded by the nucleotide sequence shown in
7 Figure 4 or a variant or fragment thereof.
8
- 9 18. The purification tag according to any one of
10 claims 15 to 17 wherein the sortase gene
11 product comprises amino acids 26 to 171 of the
12 SrtA sequence shown in Figure 4 or a variant or
13 fragment thereof.
14
- 15 19. A method of inducing and/or enhancing an immune
16 response to an antigen of interest, the method
17 comprising administering the antigen of
18 interest with a sortase, e.g srtA, gene
19 product.
20
- 21 20. The method according to claim 19, wherein the
22 sortase gene product is a Staphylococcus aureus
23 srtA gene product.
24
- 25 21. The method according to claim 19 or claim 20
26 wherein the sortase gene product is encoded by
27 the nucleotide sequence shown in Figure 4 or a
28 variant or fragment thereof.
29
- 30 22. The method according to any one of claims 19 to
31 21 wherein the sortase gene product comprises

1 amino acids 26 to 171 of the SrtA sequence
2 shown in Figure 4 or a variant or fragment
3 thereof.
4

1/11



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A B C D E F G H

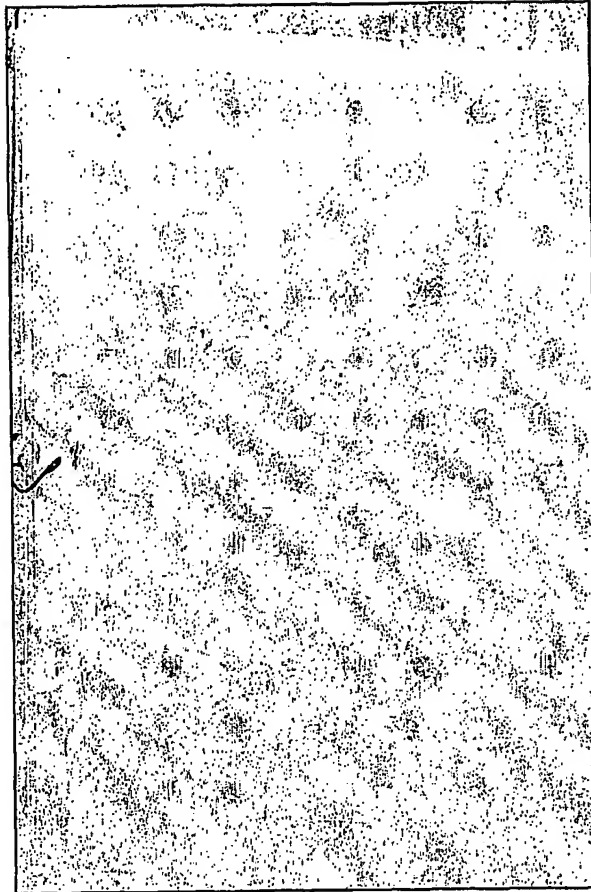
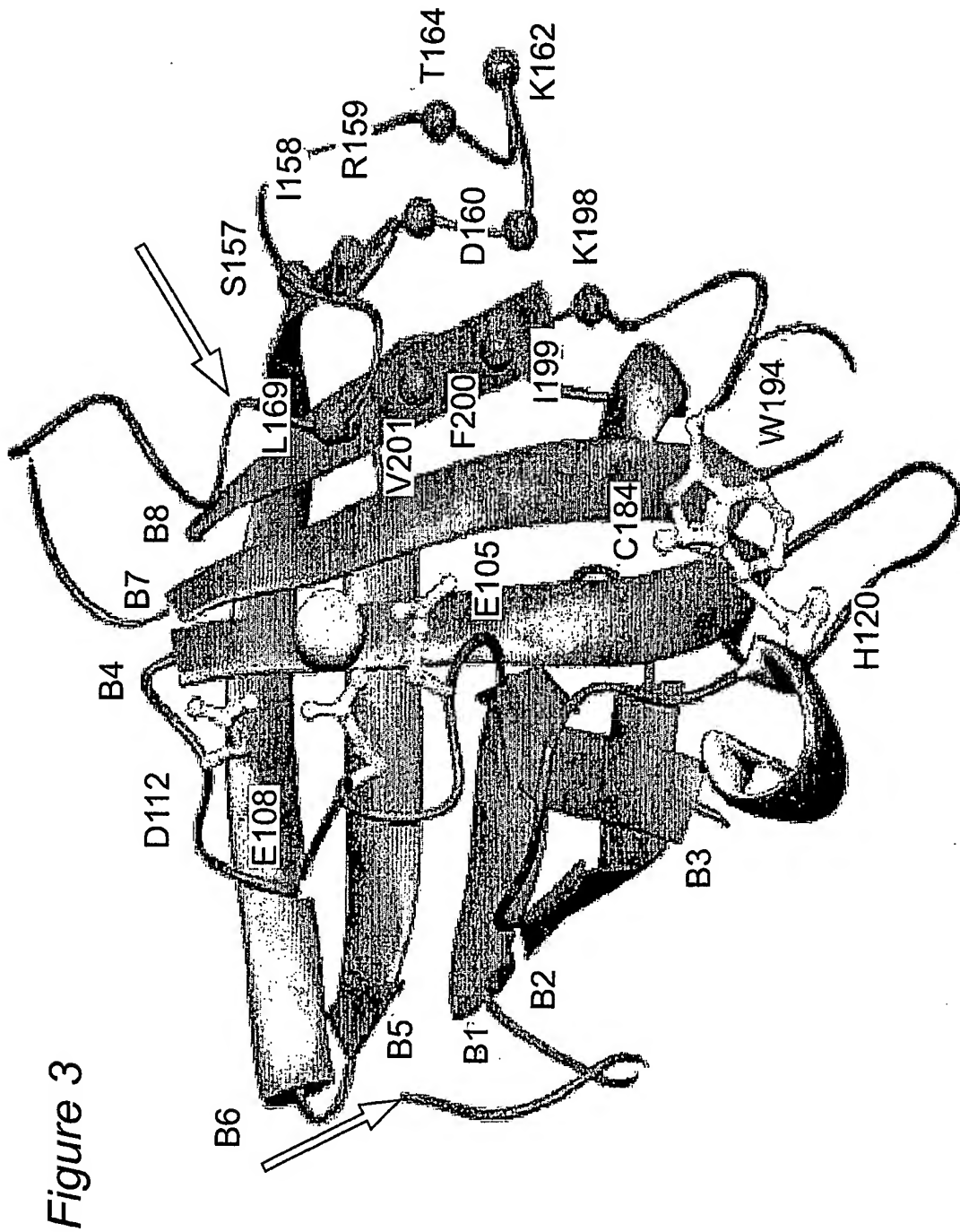


Figure 2

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ATGAGAGGATCGCATCACCATCACCATCACGGATCTAAACCACATATCGATAATTATCTT 60
TACTCTCCTAGCGTAGTGGTAGTGGTAGTGCCTAGATTTGGTGTATAGCTATTAATAGAA
M R G S H H H H H H G S K P H I D N Y L

CACGATAAAGATAAAGATGAAAGGATTGAACAATATGATAAAAAATGTAAAAGAACAGGCG 120
GTGCTATTTCTATTTCTACTTTCCTAACTTGTATATACTATTTTACATTTTCTGTCCGC
H D K D K D E R I E Q Y D K N V K E Q A

AGTAAGGATAAAAAGCAGCAAGCTAAACCTCAAATTCGAAAAGATAAATCGAAAAGTGGCA 180
TCATTCCTATTTTTCGTCGTTTCGATTTGGAGTTTAAAGGCTTCTATTTAGCTTTCACCGT
S K D K K Q Q A K P Q I P K D K S K V A

GGCTATATTGAAATTCCAGATGCTGATATTAAAGAACCAGTATATCCAGGACCAGCAACA 240
CCGATATAACTTTAAGGTCTACGACTATAATTTCTTGGTCATATAGGTCCTGGTCGTTGT
G Y I E I P D A D I K E P V Y P G P A T

CCTGAACAATTAAATAGAGGTGTAAGCTTTCGAGAAGAAAATGAATCACTAGATGATCAA 300
GGACTTGTAAATTTATCTCCACATTCGAAACGTCTTCTTTTACTTAGTGATCTACTAGTT
P E Q L N R G V S F A E E N E S L D D Q

AATATTTCAATTGCAGGACACACTTTTCATTGACCGTCCGAACTATCAATTTACAAATCTT 360
TTATAAAGTTAACGTCCTGTGTGAAAGTAACTGGCAGGCTTGATAGTTAAATGTTTAGAA
N I S I A G H T F I D R P N Y Q F T N L

AAAGCAGCCAAAAAAGGTAGTATGGTGTACTTTAAAGTTGGTAATGAAACACGTAAGTAT 420
TTTCGTCGGTTTTTTTCCATCATACCACATGAAATTTCAACCATTACTTTGTGCATTCATA
K A A K K G S M V Y F K V G N E T R K Y

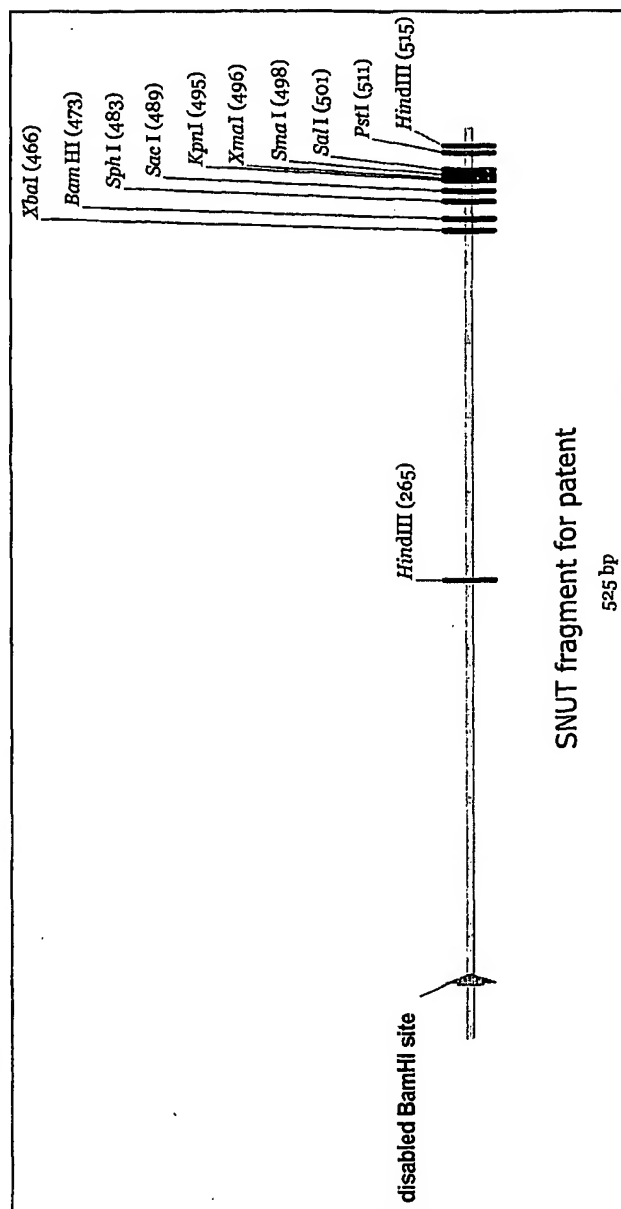
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TTTTACTGTTTCATATTCTCTACAATTCGGATGTCTACATCTTCAAGATCTACCTAGGCGT
K M T S I R D V K P T D V E V L D G S A

TGCGAGCTCGGTACCCCGGGTCGACCTGCAGCCAAGCTTAATTAG 525
ACGCTCGAGCCATGGGGCCAGCTGGACGTCGGTTCGAATTAATC
C E L G T P G R P A A K L N *

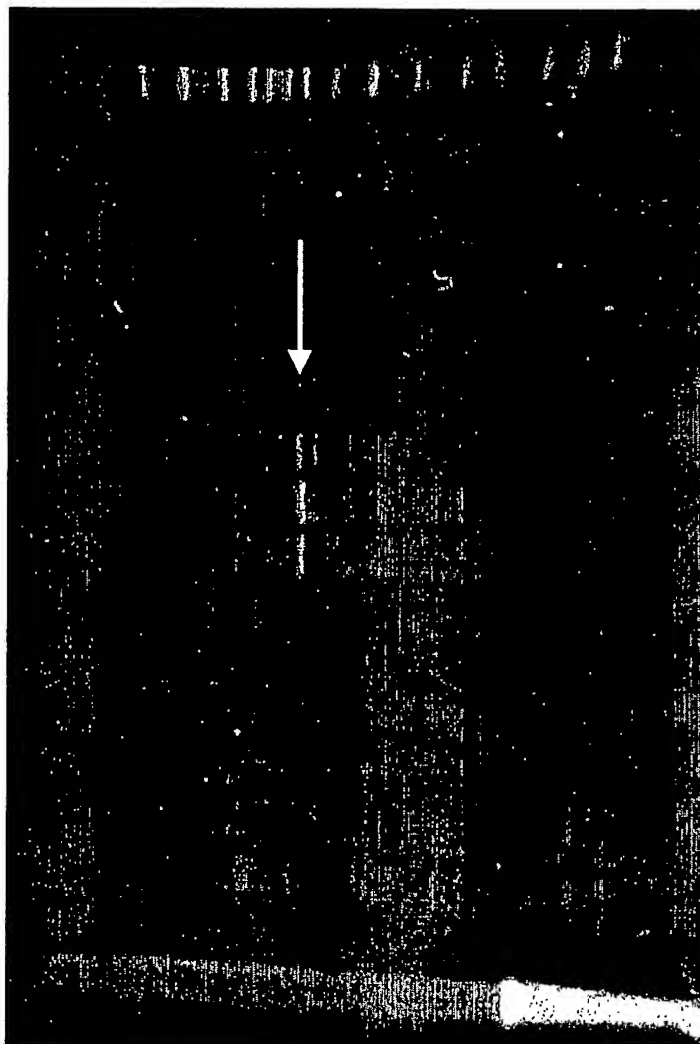
Figure 4a

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Figure 4b



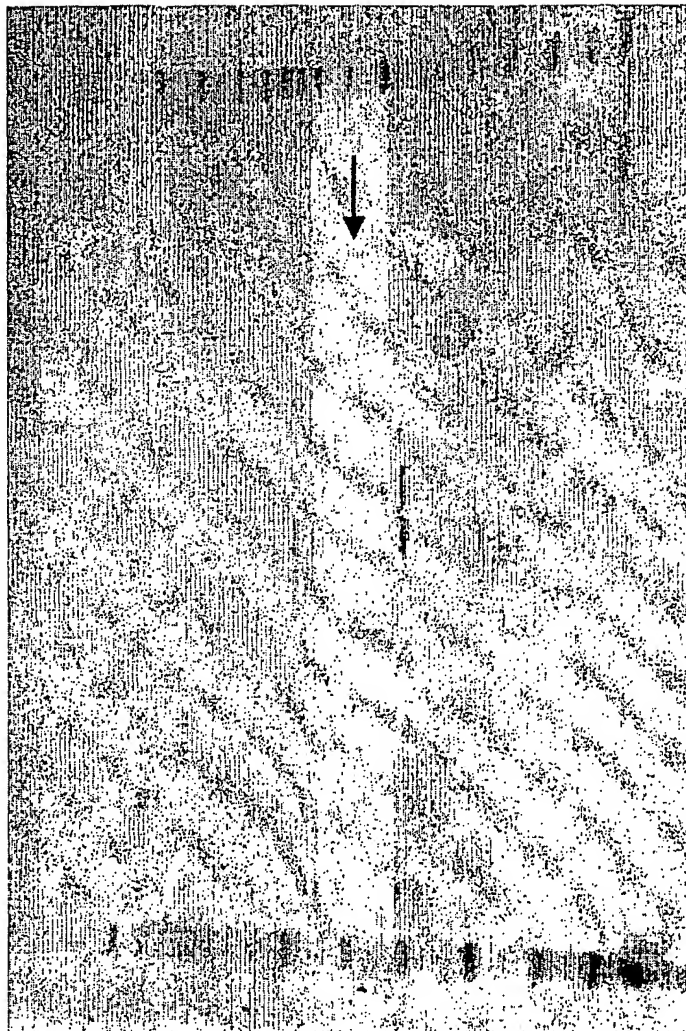
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M

Figure 5a

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M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 M

Figure 5b

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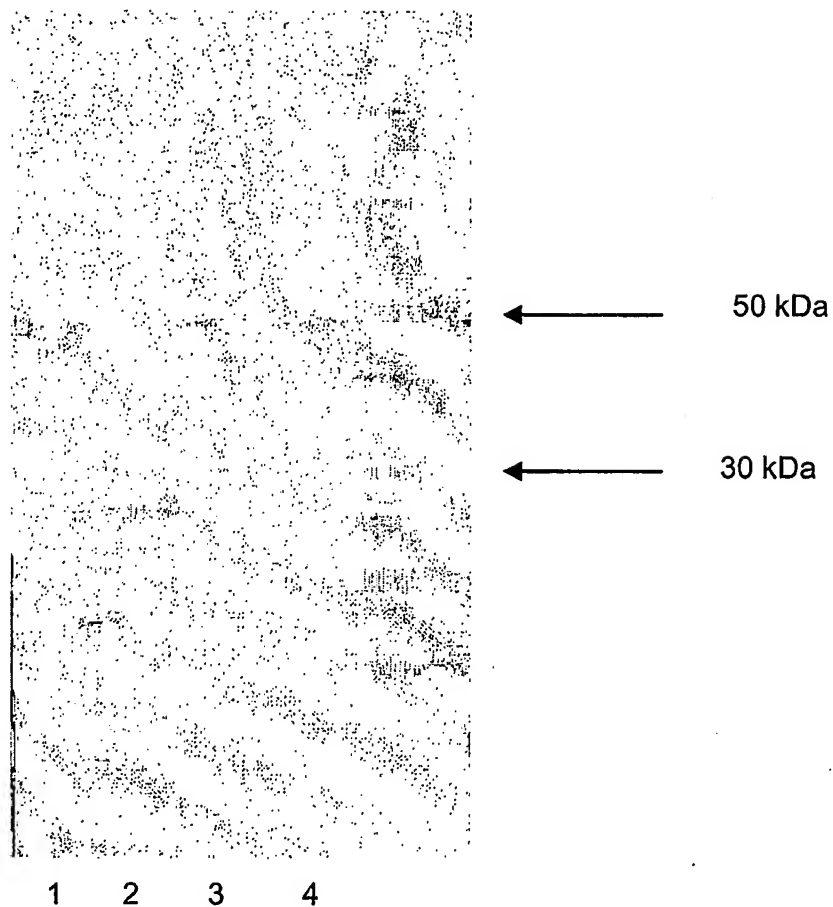


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 5c

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Figure 6



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Figure 7A

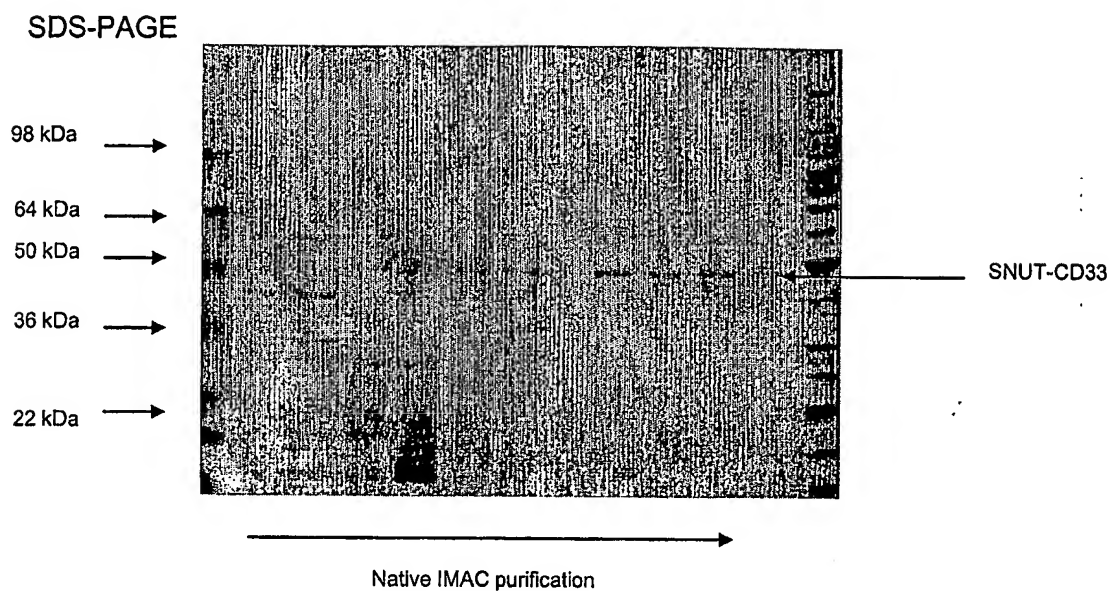
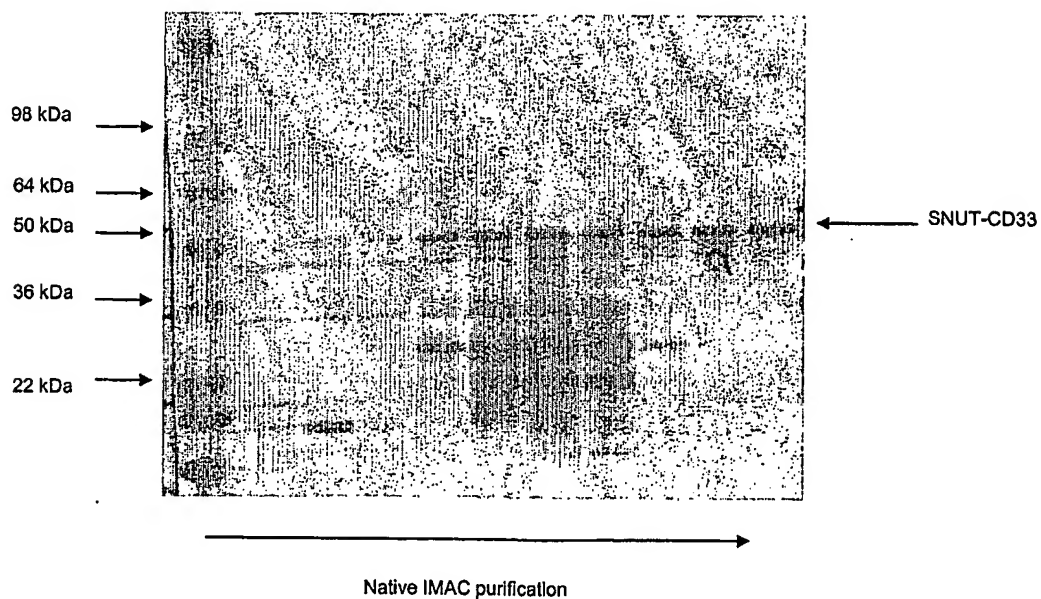


Figure 7B

Western Blot



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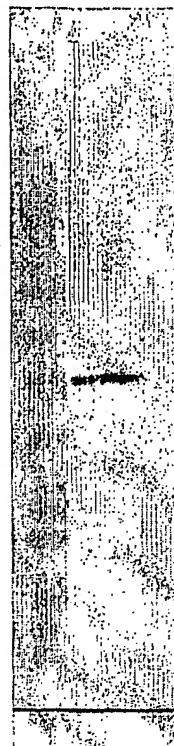
Figure 8A

Figure 8B

98 kDa →
64 kDa →
50 kDa →
36 kDa →
22 kDa →



Anti-histag
antibody



Anti-SNUT
antibody

Figure 8C

SNUT →

